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Understanding age-induced cortical porosity in women: Is a negative BMU balance in quiescent osteons a major contributor?☆

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ABSTRACT

Cortical bone is remodeled by intracortical basic multicellular units (BMUs), whose end result can be observed as quiescent osteons in histological sections. These osteons offer a unique opportunity to investigate the BMU balance between the magnitude of bone resorption and subsequent bone formation at the BMU level. Our main objective was to investigate whether the latter parameters change between defined categories of osteons and with age, and to which extend these changes contribute to age-induced cortical porosity.

Cortices of iliac bone specimens from 35 women (aged 16–78 years) with a higher porosity with age were investigated. A total of 3084 quiescent osteons reflecting 75% of the intracortical pores were histological examined. The osteons diameter, pore diameter, wall thickness, prevalence and contribution to the porosity were highly variable, but unchanged with age. Next, the osteons were categorized according to whether they reflected the remodeling of existing canals (type 2Q osteons) or the generation of new canals (type 1Q osteons). Type 2Q osteons versus type 1Q osteons: (i) had more frequently a pore diameter $> 75\,\mu m$ (7.4 vs. 1.3%; $p < 0.001$); (ii) had a larger mean pore diameter (40 ± 10 vs. $25 \pm 4\,\mu m$; $p < 0.001$), osteon diameter (120 ± 21 vs. $94 \pm 21\,\mu m$; $p < 0.001$) and wall thickness (40 ± 10 vs. 35 ± 9; $p < 0.05$); (iii) had a larger contribution to the cortical porosity (29 ± 18 vs. $8 \pm 8\%$; $p < 0.001$); (iv) were more prevalent (44 ± 10 vs. $31 \pm 11\%$; $p < 0.001$); and (v) were more prevalent with age.

Collectively, this study demonstrates that quiescent osteons with age more frequently result from remodeling of existing canals, which in some cases had a more negative BMU balance. Still, the osteons showed no overall age-related change in their pore diameter i.e. BMU balance. In contrast to conventional wisdom, these data show that non-quiescent pores, not pores of quiescent osteons, were the main contributor to a higher cortical porosity.

1. Introduction

Throughout life our skeleton is constantly remodeled to maintain its strength. This remodeling process is conducted by numerous microscopic units, termed bone remodeling units (BRUs) by Parfitt [1] and basic multicellular units (BMUs) by Frost [2]. According to the classical view, the bone remodeling conducted by these BMUs includes three tightly coupled phases: (i) a resorption phase, where the old mineralized bone matrix is removed by bone-resorbing osteoclasts; (ii) a reversal phase, where the eroded surface is colonized by osteoprogenitor/reversal cells that prepares the eroded surface for the subsequent bone formation; (iii) a formation phase, where new mineralized bone is formed on the eroded surfaces by bone-forming osteoblasts [1–4]. Importantly, the resorption and reversal phase was recently shown to be intermixed in BMUs [5]. Under normal conditions the BMUs remodel the skeleton, while maintaining its overall shape and

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mass [1,2].

During aging, a partly dysfunctional remodeling process causes bone loss, making the skeleton progressively more fragile and susceptible to osteoporotic fractures [6–10]. Around 80% of the fragility fractures observed in elderly occur in non-vertebral bones [11], where the cortical bone is especially critical for the bones overall strength [12–14]. Still, this important contribution of cortical bone to the skeletal strength is often overlooked [15,16]. In cortical bone, the age-induced bone loss is observed as both a higher cortical porosity and a lower cortical thickness [8,17–19]. The higher cortical porosity, and likely also the cortical thinning [20], is the result of dysfunctional intracortical BMUs, expanding the size of the normal occurring intracortical pores [17,21–24]. Nevertheless, the age-induced dysfunction in the intracortical BMUs has until very recently not been investigated for decades [25], and there is an unmet need for additional studies investigating the nature of this dysfunction.

The intracortical BMUs generating new canals are classically described to include a cutting cone with bone resorbing osteoclasts excavating an intracortical canal, followed by a closing cone of bone forming osteoblasts that refill the excavated canal until only a narrow canal remains [26,27]. These narrow canals are normally part of a complex network of vascularized canals, transporting nutrients and cells within the cortex [28]. The cutting cone was recently reported to include both an initial resorption with densely packed osteoclasts excavating the canal and a secondary resorption with scattered osteoclasts widening the canal, while intermixed with osteoblastic reversal cells, i.e. osteoprogenitor cells [5]. Here, the latter phase was defined as the reversal-resorption phase [5]. Importantly, the intracortical BMUs may not only generate new canals, but do also remodel the existing canals [1,25,29–37]. Here, one may argue that all intracortical BMUs generating new canals originate from intracortical BMUs remodeling existing canals, which may branch of and form new canals as well [25,29]. In histological sections cut transversely to the intracortical canals longitudinal axis, the bone structural units completed by the intracortical BMUs can be observed as quiescent osteons [26,27]. Essentially, these osteons provide a unique insight into the BMUs magnitude of resorption and formation as it passes through the plane of the histological section [38,39]. Here, the BMUs radial magnitude of resorption is outlined by the osteon cement line, while their radial magnitude of formation correspond to the wall thickness of the bone structural units formed within the cement line [40]. Moreover, the diameter of the remaining pore reflects their imbalance between the radial magnitude of resorption and formation at the BMU level, a so-called negative BMU balance [41,42].

Since the quiescent pores of osteons are the most abundant pores in the cortex [25], these quiescent osteons have been given a lot more attention than the non-quiescent pores. Several decades ago, it was proposed that the age-induced increase in cortical porosity was, in part, the cumulative result of intracortical BMUs with a negative BMU balance [22,38]. In these studies, the magnitude of bone resorption (osteon diameter) was reported to be followed by a reduced magnitude of bone formation (wall thickness) during aging, causing an age-induced negative balance between the magnitude of resorption and formation in quiescent osteons, which accordingly enlarge the osteons pore diameter [22,38,43]. This concept is in line with a similar concept in cancellous bone, proposing that a net bone loss with each remodeling transaction, i.e. a negative BMU balance, due to a reduction in the wall thickness contributes to the loss of cancellous bone during aging and osteoporosis [44–49]. Moreover, this concept for the cortical and cancellous bone loss has repeatedly been highlighted in numerous reviews and textbooks, of which some are listed here [6,41,50–52].

Nonetheless, our recent systematic classification of all pores contributing to the cortical porosity in iliac crest specimens from 35 women, clearly demonstrated that pores of quiescent osteons only contributed minimally to the age-induced cortical porosity [25]. This question the earlier widely accepted concept that a negative BMU balance at each intracortical remodeling transaction cumulatively contributes significantly to the increased cortical porosity during aging. In order to clarify this discrepancy, the present study extended our recent systematic classification of all pores contributing to the cortical porosity [25], reexamining whether the remodeling balance between the magnitude of bone resorption and subsequent bone formation change between defined categories of quiescent osteons and with age, and to which extend these changes contribute to the age-induced cortical porosity. In contrast the previous studies [22,38], the present study: (i) included approximately three-times as many osteons within each specimen; (ii) included not only osteons within the middle two quarters of the cortex, but from the entire cortex; and (iii) categorized the osteons according to whether they reflected the generation of new canals or the remodeling of existing canals, as well as their position relative to the existing osteons in the cortex. The study is part of a larger effort to investigate age-induced bone loss, and the critical dysfunctions in the remodeling process causing this bone loss.

2. Materials and methods

2.1. Bone specimens and sectioning

The cross-sectional study was conducted on undecalcified methyl methacrylate-embedded iliac crest bone specimens taken 2 cm behind the left anterior superior iliac spine from 35 women (age 16–78 years) during a forensic examination due to a sudden usually violent death [53]. None of the women showed any clinical evidence of metabolic bone diseases, nor receiving any drugs affecting the calcium metabolism, thus considered representative of a normal population. The bone specimens were sectioned as transversely as possible to intracortical canals longitudinal axis on a heavy-duty microtome (Jung Model K). The obtained 7-μm-thick sections were either Masson's Trichrome stained [54] or immunostained for osteopontin (Paragraph 2.2). One Masson's Trichrome stained section from each bone specimen were subjected to a detailed histomorphometric analysis (Paragraph 2.3).

The study was approved by the Medical Ethical Committee Erasmus MC (2016-391) in compliance with the World Medical Association Declaration of Helsinki – Ethical Principle for Medical Research Involving Human Subjects.

2.2. Immunostaining

The methyl methacrylate sections were deplastified with a xylene/chloroform mixture and with 2-methoxyethyl-acetat, rehydrated, pretreated in 1% acetic acid, and blocked with 0.5% casein (Sigma-Aldrich, Copenhagen, Denmark) in TBS [0.05 M Tris-HCl (pH 7.6) + 0.15 M NaCl] and a avidin/biotin blocking kit (DAKO, Glostrup, DK). The sections were then incubated with biotinylated goat anti-osteopontin antibodies (BAF1433, R&D systems, Minneapolis, MN, US) diluted in Renoir Red (PD904, Biocare Medical, Concord, CA, US), which were detected with alkaline phosphatase conjugated streptavidin (016-050-084, Jackson ImmunoResearch, Suffolk, UK) and visualized with Liquid Permanent Red (DAKO, Glostrup, DK). Finally, the sections were counterstained with Mayer's hematoxylin and mounted with Aquatex.

2.3. Histomorphometric analysis

In each bone specimen, the histomorphometric analysis was conducted on both the inner and outer cortices within a 6.5 mm wide zone starting 20 mm from the superior iliac crest within a single section along the transverse plane (Fig. 1A). The investigated cortices correspond to those in transiliac biopsies [53]. The cortical-trabecular bounder was carefully outlined based on both the bones structure and the lamellae structure of the bone matrix (Fig. 1B–C); making it possible separate the hemi-osteonal remodeled trabecular bone from the osteonal remodeled cortical bone [27]. The presence of marrow cells and

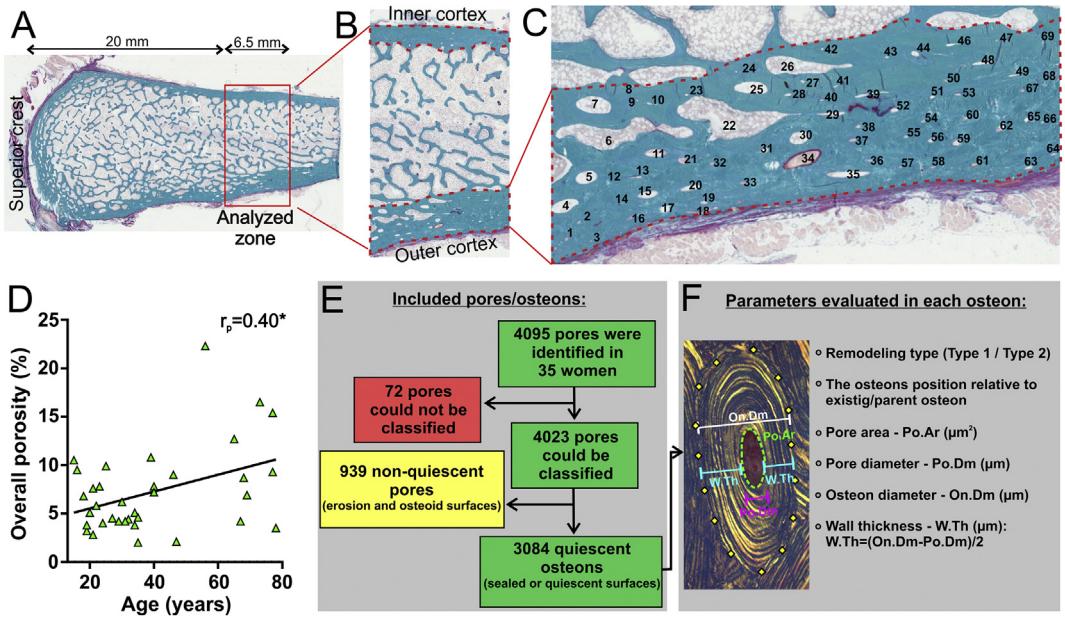

Fig. 1. The histomorphometric analysis of the intracortical pores/osteons was performed within iliac bone specimens from 35 women with an age-induced cortical porosity. A–B: The analysis was conducted on both cortices within a 6.5 mm wide zone of iliac bone specimens starting 20 mm from the iliac crest. C: Each pore within the analyzed zones was given an identification number, which were marked on a printed pore map. This made it possible to reanalyze the specific pore/osteon. D: The cortical porosity was positively correlated with age of women. Each dot represents the measurements in a given individual. The relationship between parameters was calculated using Pearson's correlation: $*p < 0.05$ and $***p < 0.001$. The curves represent the best-fitted lines for each parameter. E: 3084 of the 4023 investigable pores/osteons had quiescent or sealed bone surfaces and represented quiescent osteons. These quiescent osteons were the focus of the present study. The remaining investigable pores were non-quiescent pores (eroded and formative pores), and represented remodeling sites with a non-terminated bone remodeling F: Parameters addressed in each of the included osteons.

adipocytes could not be used to guide the bounder, as their presence appeared primary related to the size of the pores, even when deeply embedded in the cortex. All 4095 pores/osteons, including both quiescent and non-quiescent pores, observed within the defined cortices of the 35 women were given an identification number and marked on a printed map of the cortical bone (Fig. 1C) [25,30]. Polarized light made it possible to observe the surrounding lamellae and cement lines, as shown in Fig. 2. All the pores area and diameter were measured. Osteons with a sealed pore were also included in the analysis. The porosity of the cortical bones was calculated by dividing the sum of all the pore areas within a given cortex with the measured area of the cortical tissue, which was measured using a point grid. Of the investigable pores, 939 were non-quiescent pores, which were covered with eroded and osteoid (formative) surfaces. The primary focus of the present study was the 3082 quiescent osteons, which had either a sealed pore or a pore with quiescent surfaces (Fig. 1E). The diameters of these osteons were measured and their wall thicknesses calculated, as described in Fig. 1F. The diameters were measured as the diameter of the largest ball that could pass through the pore or osteon, as the pores and osteons reflect cylindrical structures in 3D, which true cross-sectional diameter has been reported to correspond to the smallest diameter of their two-dimensional profile even when they were oblique cut [39].

The osteons were classified according to their remodeling type and their resorption areas position relative to existing osteons (Fig. 1I):

i. The quiescent osteons remodeling type (type 1 or 2): Type 1Q osteons, when the resorptive area had no overlap with the pore of an existing osteon, likely representing the generation of a new canal (Fig. 2A). Type 2Q osteons, when the resorptive area overlapped with the pore of one or more existing parent osteons, likely representing the remodeling of an existing canal (Fig. 2B).
ii. The resorption area of the osteons, outlined by its cement line, location relative to the existing osteons. The type 1Q osteons was classified according to whether their resorptive area was within the cement line of an existing osteon (type $1Q_{IN}$ osteon), breaking the cement line of a single existing osteon (type $1Q_{sBK}$ osteon), breaking the cement line of multiple existing osteons (type $1Q_{mBK}$ osteon), or within the interstitial bone outside any existing osteon (type $1Q_{OUT}$ osteon). The type 2Q osteons were classified according to whether their resorptive area was within the cement line of an existing osteon (type $2Q_{IN}$ osteon), breaking the cement line of one or more osteons (type $2Q_{BK}$ osteon), or coalesced the pore of two or more existing osteons (type $2Q_{CO}$ osteon) (Fig. 2).

The analyzed sections were randomized and blinded prior to the analysis. The detailed mapping of the non-quiescent pores and quiescent osteons facilitated their specific measurements and classifications could be reviewed by the primary observer, as well as by a secondary observer. Upon disagreement, the specific measurement or classification was discussed until a consensus was reached.

2.4. Statistical analysis

The statistically significant differences between the mean parameters of type 1Q and 2Q osteons in the 35 women were identified using Wilcoxon matched-pairs signed rank test. The statistically significant differences between the mean parameters of the seven subcategories of osteons were identified using a Friedman test, followed by Dunn's posttest. The age- and porosity-association of the mean parameters in the 35 women were statistically identified using Spearman's rank correlation (r_s), while the correlations between parameters in the individual osteons were identified using linear-regressions and Pearson's correlation test (r_p). The statistically significant differences in the relative risk of type 1Q and 2Q osteons for having a pore diameter of zero or above 75 μm were identified using a χ^2 test, as the numbers of osteons in the respective biopsies were too low to compare the mean of the individual women. $P < 0.05$ was defined as statistically significant. All statistical analysis and graphical illustrations were performed using

GraphPad Prism, version 6 (GraphPad software Inc., La Jolla, CA, US).

3. Results

The present study was conducted on a defined region of the cortices within histological sections of iliac bone autopsies from 35 women (Fig. 1A–C), shown to have a higher cortical porosity with age (Fig. 1D). In the investigated iliac cortices, 4095 pores/osteons were identified, mapped and measured. The present study focused on the 3084 pores of quiescent osteons with a terminated bone remodeling, which were lined with quiescent surfaces or in some case wholly sealed. These osteons were investigated to address the dynamic range of the individual BMUs extent of bone resorption (osteon diameter), bone formation refilling the resorbed space (wall thickness) and the diameter of the remaining pore, reflecting the BMU balance between the resorption and formation. Furthermore, whether these activities were altered in given sub-

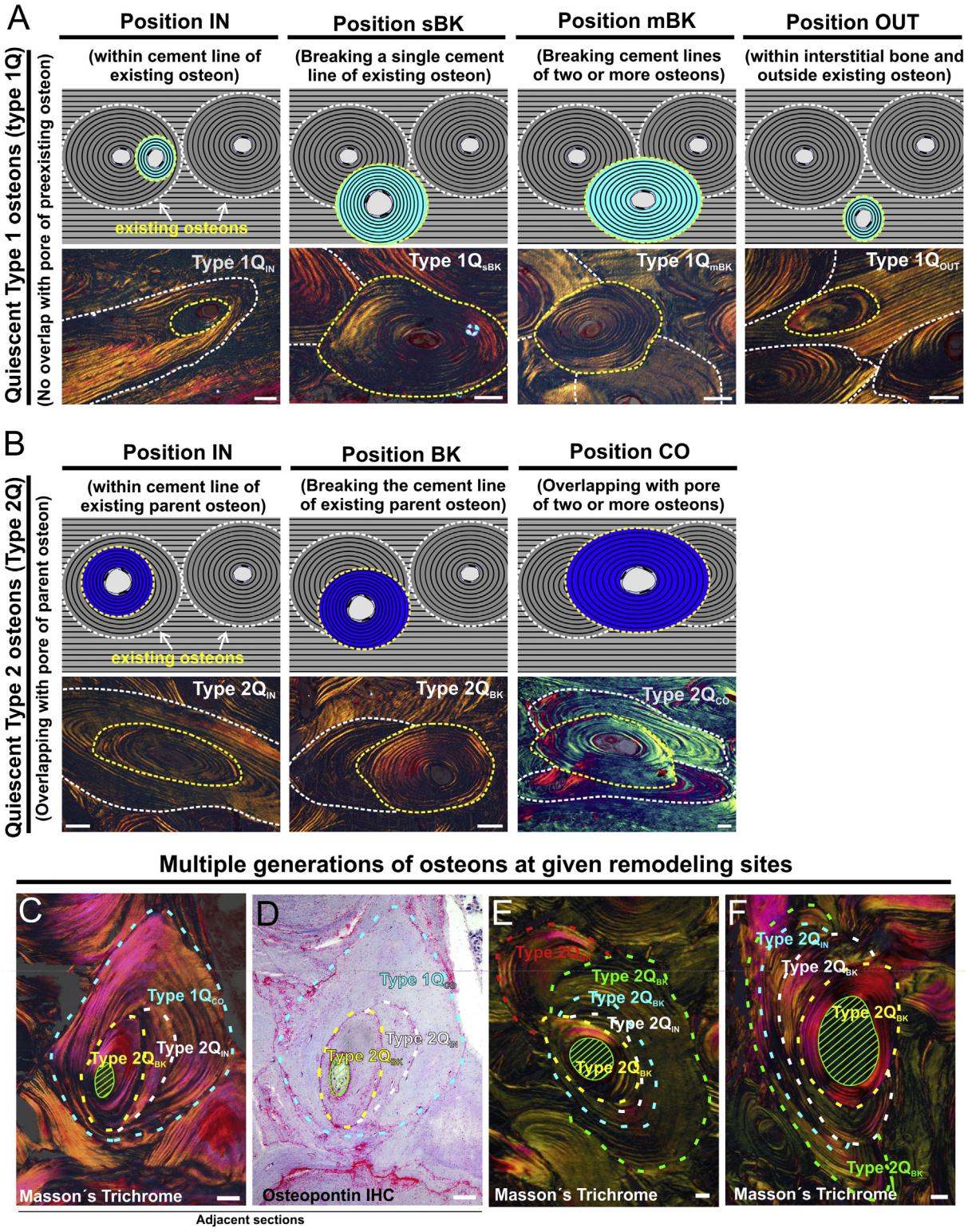

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Fig. 2. Histological appearance of type 1 and type 2 quiescent osteons, and their respective different positions relative to the existing parent osteons in Masson's Trichrome stained sections subjected to polarized light highlighting the lamella structure (A–C, E–F) and osteopontin immunohistochemical (IHC) stained sections (D). A: The osteons having a resorption area, outlined by the cement line (yellow hatched line), that showed no overlap with the pores of an existing osteons were defined as type 1 osteons. These osteons reflected remodeling sites generating new canals. B: The osteons having a resorption area that overlapped with the pore of their parent osteon were defined as type 2 osteons. These osteons reflected the remodeling of existing canals. Both type 1 and 2 osteons were then further subtyped according to their resorption spaces position relative to the preexisting/parent osteons cement line (white hatched lines) and pore. Position IN: The resorption area of the osteon remained within the cement line of the existing/parent osteon. Position BK: The resorption area of the osteon break the cement line of the existing/parent osteon. For type 1Q osteons position sBK break a single cement line, while position mBK break cement lines of multiple osteons. Position CO: The resorption area of type 2Q osteons overlaps with the pores of two or more parent osteons. Position OUT: The resorption area of the type 1Q osteon was within the interstitial bone and had no overlap with an existing osteon. C–F: Three examples of multiple generations of type 2 osteons overlapping the pore each other at given remodeling sites. The osteons are outlined by their cement line (hatched lines), which could both be observed in Masson's Trichrome stained sections (B, C, E, F) and osteopontin immunohistochemical (IHC) stained (D) section adjacent to C. Note that the cement lines of the youngest (yellow hatched line), parent (white hatched line), grandparent (blue hatched line) and great-grandparent (green hatched line) osteons can be observed, making it possible to detect four generations osteons at the same site. Pores are marked green. Scale bars are 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

categories of osteons, and whether they were affected by aging and in cortices with a higher cortical porosity. Finally, the remaining 939 non-quiescent pores, covered with eroded and osteoid surfaces, were analyzed as an alternative contributor to the higher cortical porosity with age.

3.1. Classification of osteons

As described in Paragraph 2.3 and Fig. 2A–B the quiescent osteons were classified according to their remodeling type and detailed position relative to existing osteons, in accordance with our previous reported classification [25,30]. Importantly, type 1 osteons reflect the remodeling activities of BMUs generating new canals, while type 2 osteons reflect the remodeling activities of BMUs modulating existing canals within the plain of the histological section. This means that the starting points of the two types of remodeling are very different, and that type 1 osteons may over time be converted into type 2 osteons, if the generated pore is remodeled by another BMU. The resorptive area of both type 1 and 2 osteons had very different positions relative to the pores and cement lines of existing osteons. This difference in their position was incorporated into the osteons sub-classification (Fig. 2A–B and Paragraph 2.3). Note that the type 2_{CO} osteons reflect the activities of BMUs coalescing two or more existing canals, whereas type 2_{IN} and 2_{BK} osteons only remodeled a single existing canals (Fig. 2B). Moreover, the type 2 osteons may reflect the result of multiple generations of BMUs, as we often observed multiple type 2 osteons on top of each other (Fig. 2C–F). This provided a unique opportunity to trace the repeated remodeling of a given remodeling site several generations back, as shown in Fig. 2C–F, showing up to four generations of osteons at the same site. This means that the dimensions of type 2 osteons are affected by the pore size of its parent, which in turn is affected by the pore size of the grandparent osteon and so forth.

3.2. Pore diameters, osteon diameters and wall thicknesses of individual osteons

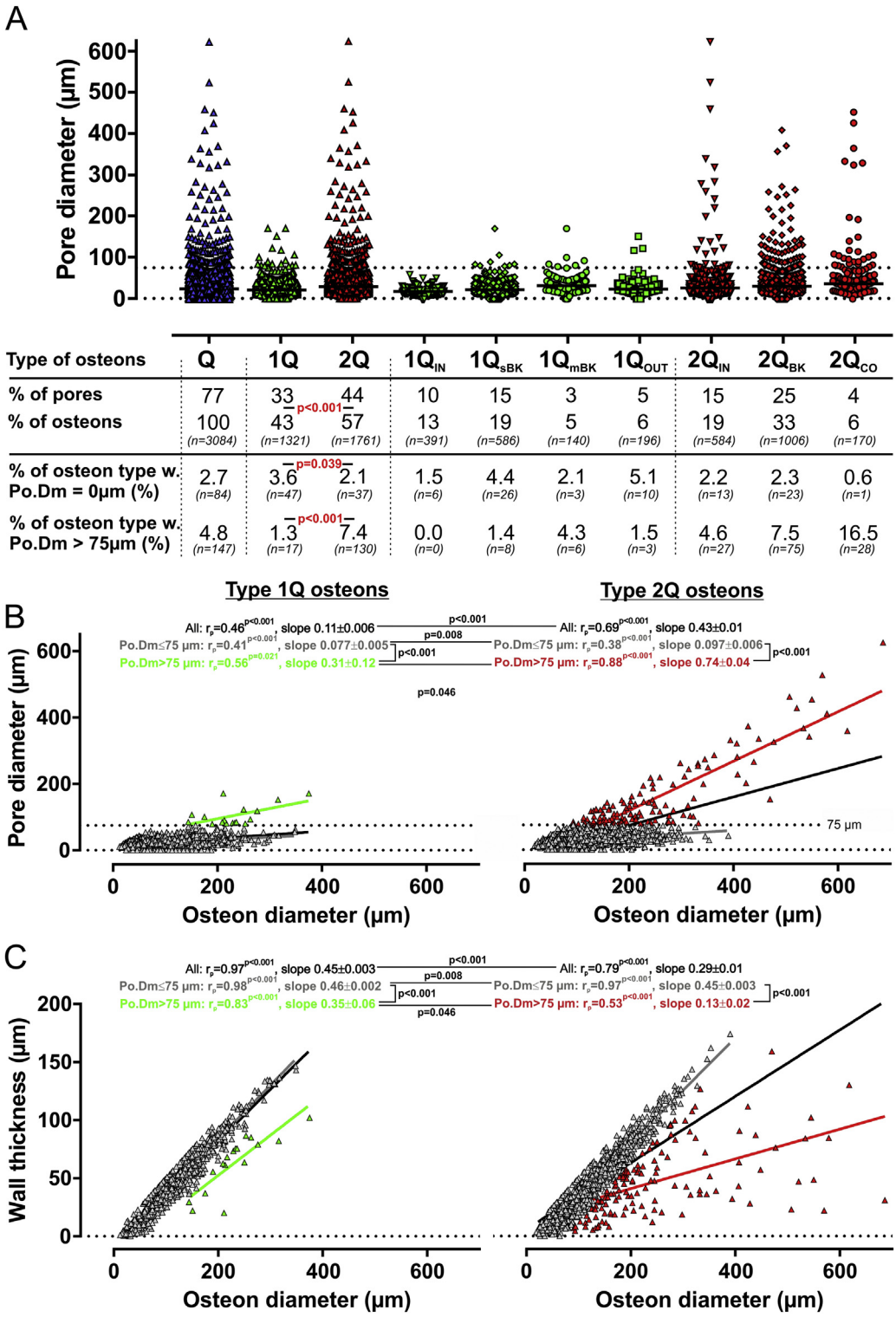
The pore diameters of individual osteons were shown to be highly variable, ranging from 0 to 624 μm (Fig. 3A). Of these osteons, 4.8% had a pore diameter above 75 μm and 2.7% had a sealed pore (Po.Dm = 0 μm). The different categories of osteons had a very different prevalence and distribution of pore diameters. All categories had some osteons with a sealed pore. In general, the type 2 osteons were more prevalent, had a larger and wider range of pore diameters, and therefore more pores with a diameter above 75 μm and less sealed pores compared to type 1 osteons (Fig. 3A). Indeed, only type 2 osteons had pore diameters above 200 μm. Type 1_{sBK} osteon was the most prevalent type 1 osteon, whereas type 2_{BK} osteon was the most prevalent type 2 osteon (Fig. 3A). The individual osteons diameter and wall thickness were also shown to be highly variable, ranging from 21 to 686 μm and 2–174 μm (Fig. 3B–C). Only type 2 osteons had diameters above 400 μm.

Linear-regression analysis of the osteon diameter, as a predictor of the pore diameter, revealed that type 2 osteons have a significant higher slope (linear coefficient β) than type 1 osteons. This supports that the diameter of type 2 osteons had a bigger influence on the pore diameter than in type 1 osteons (Fig. 3B). This difference between type 2 and 1 osteons was primary due to the abundance of type 2 osteons with pore diameter > 75 μm, whereas the type 2 osteons with a pore diameter < 75 μm actually had a similar relationship between the osteon and pore diameter as the type 1 osteons (Fig. 3B). In line with this, the linear-regression analysis of the osteon diameter, as a predictor of the wall thickness, revealed that type 2 osteons have a smaller slope than type 1 osteons. This supports that the diameter of type 2 osteons had a poorer influence on the wall thickness compared to type 1 osteons (Fig. 3C). Again, this was primary due to the type 2 osteons with a pore diameter > 75 μm, whereas the type 2 osteons with a pore diameter < 75 μm actually had similar a relationship between their osteon diameter and wall thickness as the type 1 osteons (Fig. 3C). These data highlight that precisely the type 2 osteons with a pore diameter > 75 μm have a poorer correlation between the osteon diameter and the wall thickness, and a better correlation between the osteon diameter and the pore diameter compared to all the other osteons. Most of these type 2 osteons with a pore diameter > 75 μm reflected the cumulative outcome of multiple remodeling transactions at a given remodeling site, each including the remaining pore of the parent osteon within its resorption space, as illustrated in Fig. 2C–F.

Importantly, the prevalence of these osteons with a pore diameter > 75 μm, as well as their contribution to the total pore area, was statistically significant higher in bone specimen's with a higher cortical porosity, but not in aging women (Fig. 4). Taken together, the present analysis of the individual osteons highlights that especially some of the type 2 osteons have enlarged pores, resulting from enlarged resorption areas (osteon diameter) including the pore of its parent osteon, which were insufficiently refilled (wall thickness). However, one should note that these osteons with a pore diameter > 75 μm only contributed to 1% (range 0–4%) of the cortical porosity, while non-quiescent pores with a pore diameter > 75 μm contributed to 71% of the cortical porosity.

3.3. Osteon diameter, wall thickness and pore diameter association with age and porosity

In order to investigate whether the osteons dimensions change with age and the cortical porosity, we calculated the osteons mean diameter, wall thickness and pore diameter within each specimen and correlated these mean parameters with the women's age and the specimen's overall cortical porosity. The median number of osteons in each specimen was 82, ranking from 44 to 145. Overall, the type 2 osteons had a significant larger mean diameter, wall thickness and pore diameter than type 1 osteons (Fig. 5). The osteons mean diameter, wall thickness and pore



Type of osteons	Q	1Q	2Q	$1Q_{IN}$	$1Q_{sBK}$	$1Q_{mBK}$	$1Q_{OUT}$	$2Q_{IN}$	$2Q_{BK}$	$2Q_{CO}$
% of pores	77	33	44	10	15	3	5	15	25	4
		p<0.001								
% of osteons	100	43	57	13	19	5	6	19	33	6
	(n=3084)	(n=1321)	(n=1761)	(n=391)	(n=586)	(n=140)	(n=196)	(n=584)	(n=1006)	(n=170)
% of osteon type w. Po.Dm = 0µm (%)	2.7	3.6	2.1	1.5	4.4	2.1	5.1	2.2	2.3	0.6
	(n=84)	p=0.039 (n=47)	(n=37)	(n=6)	(n=26)	(n=3)	(n=10)	(n=13)	(n=23)	(n=1)
% of osteon type w. Po.Dm > 75µm (%)	4.8	1.3	7.4	0.0	1.4	4.3	1.5	4.6	7.5	16.5
	(n=147)	p<0.001 (n=17)	(n=130)	(n=0)	(n=8)	(n=6)	(n=3)	(n=27)	(n=75)	(n=28)

Fig. 3. The prevalence and dimensions of individual type 1 and type 2 quiescent osteons and their respective subcategories within the investigated cortices. Each dot reflects the measurement in a given osteon. A: The type 1 and type 2 osteons and their respective subcategories range of pore diameters. The black horizontal bars reflect the median. The first two rows of the table reports the percentage of the collective number pores and osteons reflecting type 1 and type 2 osteons and their respective subcategories in the investigated cortices. The third and fourth row of the table shows percentage of type 1 and type 2 osteons and their respective subcategories that have a pore diameter (Po.Dm) equal to zero and above 75 µm. Statistically significant differences between the incidence in type 1 and 2 remodeled osteons were calculated using a χ^2 test. B–C: Correlations of the individual type 1 or type 2 osteon diameters with pore diameters (B) or wall thickness (C). The type 1 and type 2 osteons are subdivided according to their pore diameters: above 75 µm (green and red dots), or below or equal to 75 µm (grey dots). The lines reflect the linear-regression between the parameters, which slopes and Pearson's correlation (r_p), and the statistically significant difference between these slopes are reported above the graphs. Note that a pore diameter above 75 µm was more prevalent for type 2 than type 1 osteons. These type 2 osteons have a better relationship between their pore and osteon diameter and a poorer relationship between their osteon diameter and wall thickness compared to all the other subgroups of osteons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diameter were also highly dependent on their sub-classification. In general, the type 2_{IN}, 2_{BK} and 2_{CO} osteons were step-wise larger in all three mean parameters (Fig. 5). This is in accordance with their definition, as a larger osteons also have a greater risk of overlapping with the cement lines and pores of existing osteons. On the other hand, one should note the mean osteon diameter of type$2Q_{CO}$ osteons is not significant larger than type $1Q_{mBK}$ or 2_{QBK} osteons, supporting that pores not just coalesce because they have a larger radial resorption i.e. osteon diameter.

When considering all osteons, no age-correlation was found with their mean diameter, wall thickness and pore diameter and the age of the women (Fig. 5). According to these correlations, the osteons´ extent of resorption (osteon diameter), subsequent refilling (wall thickness) and the balance between the two (pore diameter) appeared largely unchanged with age. On the other hand, the correlations with the overall cortical porosity of the bone specimens showed a positive correlation with the osteons pore diameter, a negative correlation with the osteons wall thickness and no correlation with the osteons diameter (Fig. 5). The same was, however, not the case when dividing the osteons into type 1 and 2 osteons. Here the type 1 osteons diameter and wall

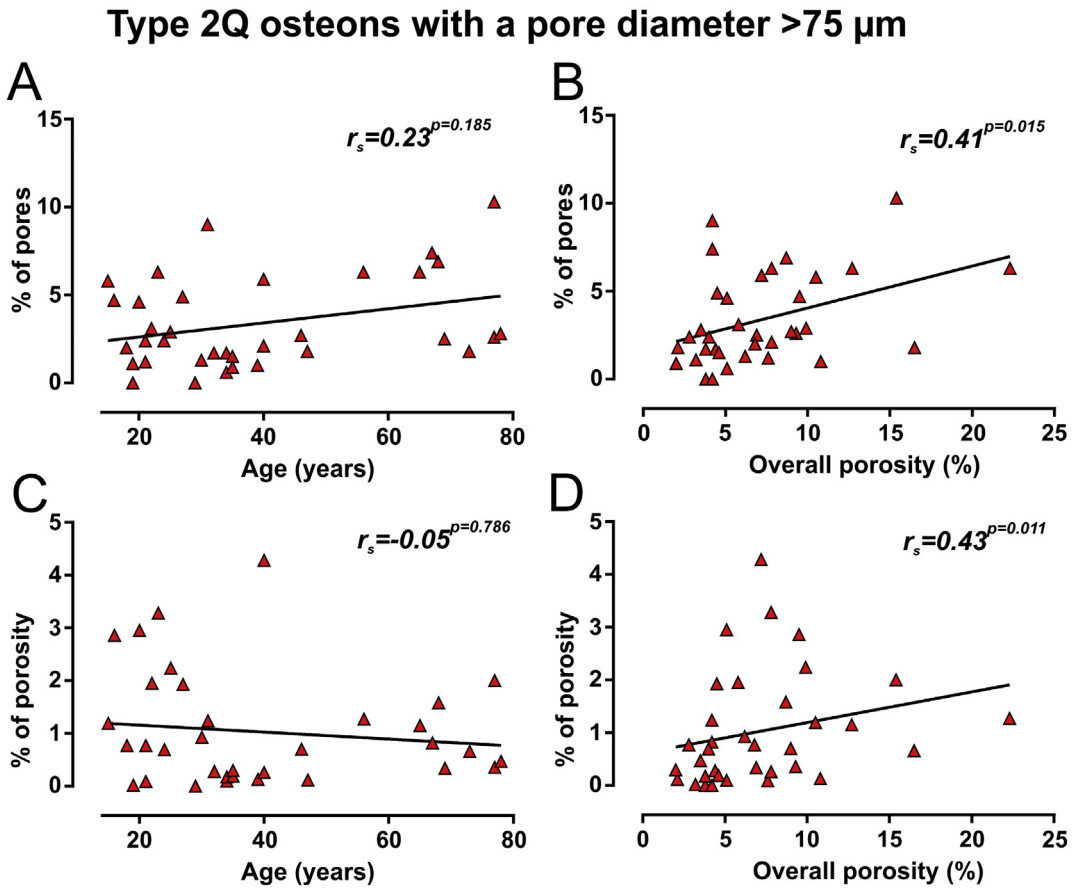


Fig. 4. Close-up on the prevalence of type 2 quiescent osteons with a pore diameter above 75 µm (A–B) and their contribution to the porosity in the iliac bone specimens of 35 women (C–D), as well as these parameters correlation with the women's age (A, C) and the iliac specimens overall cortical porosity (B, D). Each dot represents the measurements in a given individual and the curves represent the best fitted-lines. Note that nor their prevalence (A) neither contribution to the porosity (C) correlate with age, but positively with the overall cortical porosity (B, D). The parameters correlations with age or cortical porosity were calculated using Spearman's Rank correlation test (r_s).

thickness showed a negative correlation with porosity, while the pore diameter showed no correlation with porosity. Moreover, the parameters of the type 2 osteons had no correlation with porosity (Fig. 5).

3.4. Pore and osteon prevalence with age and contribution to a higher porosity

We investigated whether the osteons prevalence and contribution to the porosity in the respective bone specimens was associated with the age of the women and the bone specimens overall cortical porosity. Overall, the quiescent osteons represented 76% of the identified pores, ranging from 51 to 87%, while their pores contributed to merely 33% of the porosity, ranging from 8 to 85% (Fig. 6A–B). The osteons prevalence and contribution to the porosity showed no correlation with the women's age, but a negative correlation with the specimen's cortical porosity (Fig. 6A–B). Collectively, these data imply that the pores of quiescent osteons only have a negligible contribution to the higher cortical porosity during aging.

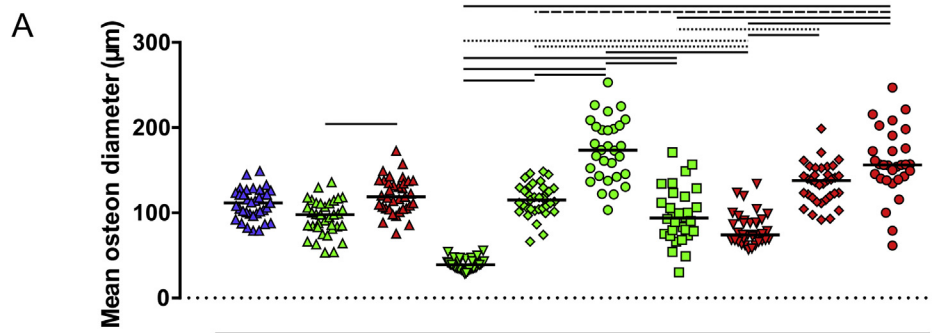
When separating type 1 and 2 osteons, type 2 osteons were shown to have a much larger contribution to the porosity than type 1 osteons (26% versus 5%). Moreover, the prevalence of type 2 osteons had a positive correlation with age, predominantly due to a higher prevalence of type 2_{IN} and 2_{CO} osteons, while the prevalence of type 1 osteons had a negative correlation with age, predominantly due to a lower prevalence of type 1_{sBK} and 1_{OUT} osteons (Fig. 6A–B). These age-related changes in the prevalence of type 1 and 2 osteons implies that the remodeling of existing pores become more prevalent with age, while the generation of new becomes less prevalent. The prevalence of type 1 and 2 osteons showed a negative correlation with the specimen's cortical porosity (Fig. 6B).

On the other hand, the non-quiescent pores prevalence and contribution to the porosity were shown to have a strong positive correlation with the specimen's cortical porosity (Fig. 6B). The median number of these pores detected in each specimen were 27, ranking from 11 to 53. Here, one should note that these non-quiescent pores had a median contribution to the cortical porosity of 67% (range 14–91%), as they were in general larger than the pores of quiescent osteons.

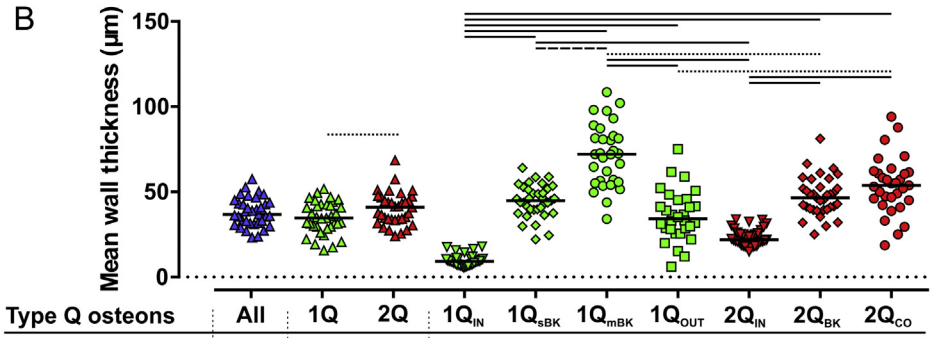
4. Discussion

In order to investigate the concept that age-induced increase in cortical porosity is the cumulative result of intracortical BMUs with a negative BMU balance [22,38], we investigated the magnitude of resorption, formation and the remaining pore, i.e. BMU balance, of 3084 quiescent osteons in ilia of 35 women. Moreover, we investigated their contribution to the cortical porosity relative to the contribution of non-quiescent pores. The rationale behind the investigated concept is that the minute net bone loss generated by each remodeling transaction with a negative BMU balance form quiescent osteons with an enlarged pore, which cumulatively leads to a higher cortical porosity with age [22,38,41,42]. This is in line with the notion that enlarged and coalescing pores, not a higher pore density, is responsible for the higher cortical porosity with age [23–25,41].

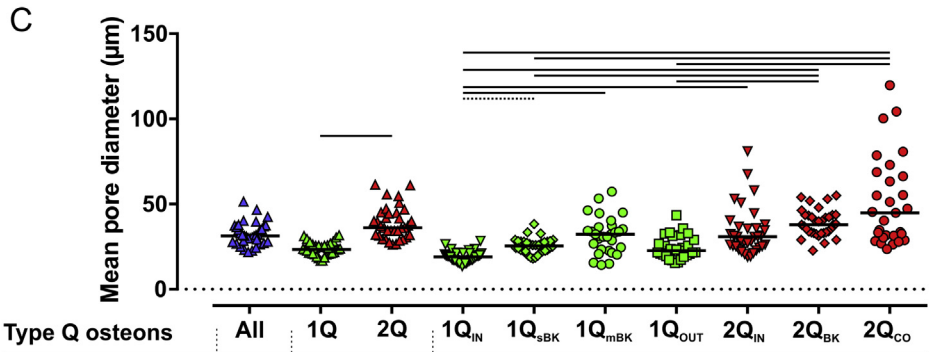
Taken together, we demonstrate that a negative BMU balance by



Type Q osteons		All	1Q	2Q	$1Q_{IN}$	$1Q_{sBK}$	$1Q_{mBK}$	$1Q_{OUT}$	$2Q_{IN}$	$2Q_{BK}$	$2Q_{CO}$
Correlation with age	r_s	-0.16	-0.27	-0.26	-0.06	-0.26	-0.09	-0.20	-0.13	-0.19	-0.21
	p	0.352	0.112	0.133	0.723	0.138	0.614	0.297	0.456	0.281	0.267
Correlation with porosity	r_s	-0.19	**-0.35**	-0.14	0.00	**-0.41**	-0.24	-0.30	0.02	-0.14	0.05
	p	0.279	**0.040**	0.427	0.985	**0.013**	0.191	0.102	0.901	0.444	0.794



Type Q osteons		All	1Q	2Q	$1Q_{IN}$	$1Q_{sBK}$	$1Q_{mBK}$	$1Q_{OUT}$	$2Q_{IN}$	$2Q_{BK}$	$2Q_{CO}$
Correlation with age	r_s	-0.21	-0.30	-0.24	-0.07	-0.24	-0.06	-0.18	-0.22	-0.23	-0.03
	p	0.224	0.077	0.162	0.708	0.159	0.743	0.337	0.206	0.176	0.886
Correlation with porosity	r_s	**-0.34**	**-0.35**	*-0.31*	-0.13	**-0.43**	-0.24	-0.30	-0.07	**-0.33**	-0.13
	p	**0.043**	**0.040**	*0.073*	0.445	**0.010**	0.191	0.102	0.694	**0.050**	0.488



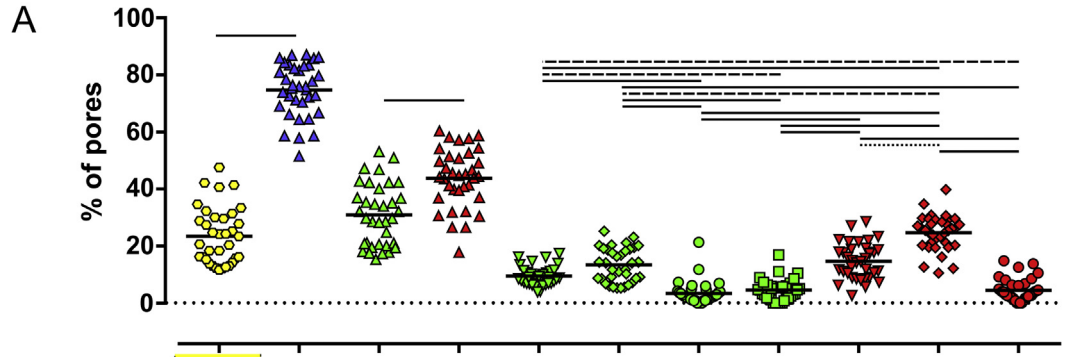
Type Q osteons		All	1Q	2Q	$1Q_{IN}$	$1Q_{sBK}$	$1Q_{mBK}$	$1Q_{OUT}$	$2Q_{IN}$	$2Q_{BK}$	$2Q_{CO}$
Correlation with age	r_s	0.05	-0.09	-0.07	0.11	-0.06	0.15	-0.09	-0.04	-0.02	-0.03
	p	0.790	0.595	0.675	0.540	0.733	0.424	0.655	0.851	0.894	0.885
Correlation with porosity	r_s	**0.34**	0.10	*0.30*	*0.30*	0.09	0.21	-0.05	0.04	*-0.32*	**0.42**
	p	**0.043**	0.577	*0.076*	*0.075*	0.618	0.258	0.800	0.812	*0.061*	**0.013**

Fig. 5. The mean osteon diameter (A), wall thickness (B) and pore diameter (C) of the type 1 and type 2 quiescent osteons and their respective subcategories, and their correlation with age and cortical porosity. Each dot reflects the mean diameter/thickness in a given woman (n = 35), while the black horizontal bars reflect the median of the 35 women. Statistically significant differences between the type 1 and 2 osteons were calculated by Mann-Whitney test, while the statistical significant differences between their subcategories were calculated using a Friedman test (overall: $p < 0.001$), followed by a Dunn's posttest: Dotted bar, $p < 0.05$; Hatched bar, $p < 0.01$; and unbroken bar, $p < 0.001$. The parameters correlations with age or cortical porosity were calculated using Spearman's Rank correlation test (r_s). The significant correlations are in bold. Note that all three parameters are increased in type 2 versus type 1 osteons; that none of the parameters correlated with age; and that some of the parameters correlated with the cortical porosity.

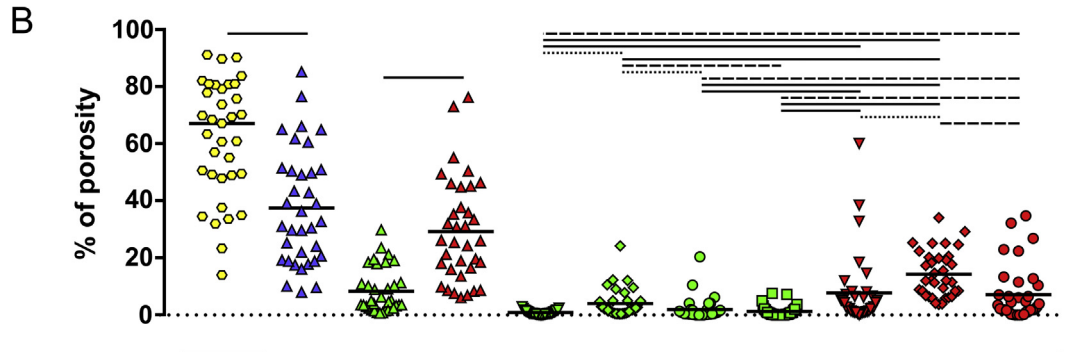
each remodeling transaction may lead to the generation of quiescent osteons with an enlarged pore, and that the prevalence of these osteons had a positive correlation with the overall cortical porosity, but no correlation with age. These enlarged quiescent osteons, however, only had a minor contribution to the cortical porosity (1%) compared to non-quiescent pores (71%) in ilia of women. Moreover, the quiescent osteons mean diameter, wall thickness and pore diameter were in general unchanged with age. Collectively, these data questions the significance of quiescent osteons with a negative BMU balance to the higher cortical porosity with age [25], as discussed below and summarized in Fig. 7.

4.1. Type 2Q osteons - the cumulative end result of multiple intracortical BMUs

Our new classification and analysis of quiescent osteons, extending



		Non-Q pores	All	1Q	2Q	$1Q_{IN}$	$1Q_{sBK}$	$1Q_{mBK}$	$1Q_{OUT}$	$2Q_{IN}$	$2Q_{BK}$	$2Q_{CO}$
			Type of quiescent osteons									
Correlation with age	r_s	0.19	-0.21	**-0.48**	**0.46**	-0.08	**-0.48**	-0.12	**-0.66**	**0.35**	0.05	**0.41**
	p	0.266	0.236	**0.004**	**0.016**	0.633	**0.004**	0.483	**<0.001**	**0.040**	0.761	**0.003**
Correlation with porosity	r_s	**0.56**	**-0.55**	**-0.40**	-0.14	*-0.30*	**-0.38**	-0.22	-0.21	-0.05	*-0.32*	-0.03
	p	**<0.001**	**<0.001**	**0.018**	0.422	*0.085*	**0.026**	0.202	0.226	0.772	*0.061*	0.846



		Non-Q pores	All	1Q	2Q	$1Q_{IN}$	$1Q_{sBK}$	$1Q_{mBK}$	$1Q_{OUT}$	$2Q_{IN}$	$2Q_{BK}$	$2Q_{CO}$
			Type of quiescent osteons									
Correlation with age	r_s	0.22	-0.22	*-0.33*	-0.17	-0.21	**-0.44**	-0.17	**-0.59**	0.04	-0.27	0.10
	p	0.204	0.196	*0.055*	0.331	0.218	**0.008**	0.339	**<0.001**	0.84	0.122	0.569
Correlation with porosity	r_s	**0.59**	**-0.57**	**-0.66**	**-0.50**	**-0.72**	**-0.69**	**-0.47**	**-0.54**	**-0.36**	**-0.50**	-0.21
	p	**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	**<0.001**	**0.005**	**<0.001**	**0.033**	**0.003**	0.220

Fig. 6. Prevalence (A) and contribution to the cortical porosity (B) of non-quiescent pores (non-Q pores) and quiescent type 1 and 2 osteons, including their respective subcategories, as well as their correlation with age and the overall cortical porosity. Each dot reflects the percentage in a given woman, while the black horizontal bars reflect the median of the 35 women. Statistically significant differences between the type 1 and 2 osteons were calculated by Mann-Whitney test, while the statistically significant differences between their subcategories were calculated using a Friedman test (overall: $p < 0.001$), followed by a Dunn's posttest: Dotted bar, $p < 0.05$; Hatched bar, $p < 0.01$; and unbroken bar, $p < 0.001$. The percentages correlations with age or cortical porosity were calculated using Spearman's Rank correlation test (r_s). The significant correlations are in bold. Note that the prevalence of type 2 osteons increase with age, while the prevalence of type 1 osteons decline with age; and that the osteons prevalence and contribution to the total pore area have a negative correlation with the cortical porosity.

our recent established classification criteria [25,30], highlights that the intracortical BMUs not only generate new intracortical canals (type 1Q osteons), but in most cases remodel existing canals (type 2Q osteons). The notion that intracortical BMUs remodel existing canals is not new, but often overlooked. For decades, numerous sporadic studies from different groups have repeatedly shown that this is the case [29,32–37,55,56]. These studies are, however, either purely descriptive or selectively focused on given sub-categories of osteons. The new classification used in this study embraces and combines these sub-categories of quiescent osteons, providing a common classification of the type 2Q osteons and their sub-categories. Here, the type $2Q_{IN}$ osteons correspond to the double-zoned osteons [33,35], type II osteons [29,36] and osteons within old osteons [32,34,37,55], previously reported to be within the cement line of an existing osteon. Type $2Q_{BK}$ osteons correspond to so-called "breakout zones" [29,37], where the new osteon break the cement line of an existing osteon. These zones were reported to be "encountered with great frequency" in osteons 3-dimensionally traced in serial cross-sections of cortical bone from dogs, baboons and a human [37], and observed in human femur using synchrotron radiation μCT scanning [29]. The type $2Q_{CO}$ osteons may, in part, reflect so-called composite osteons, which have been reported to be generated by the coalescence of multiple osteons and mirror 83% of the giant pores with a pore diameter above 385 μm [56]. Nonetheless, the quiescence of these composite osteons has been questioned [25], since the illustration of the investigated composite osteons actually seem to have new eroded surfaces [56], meaning that according to our criteria they should have been classified as eroded type 2_{CO} pores [25,30].

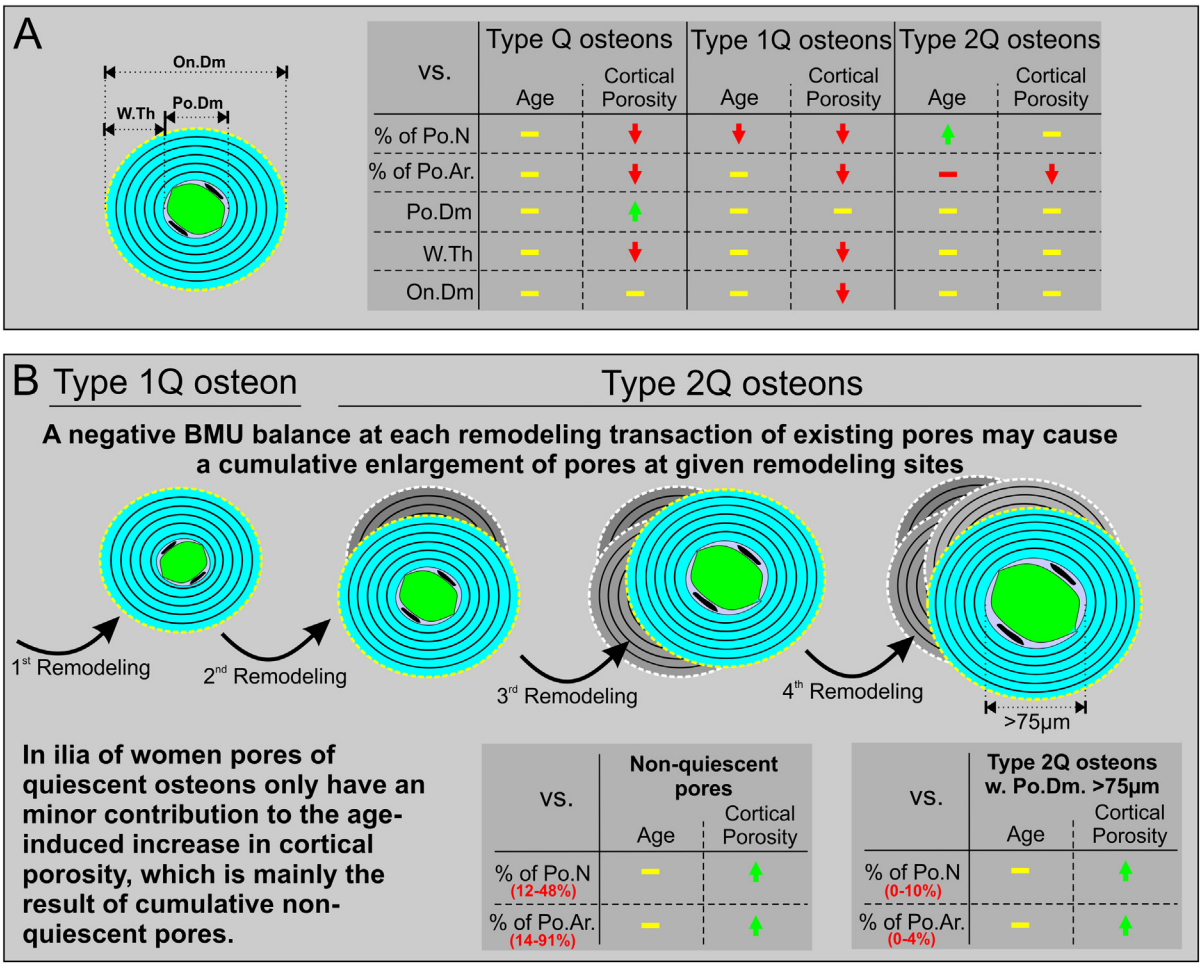


Fig. 7. Summarizing tables (A, B) and model (B), highlighting that recurring remodeling transactions with a negative BMU balance at given remodeling sites may cumulative generate type 2 osteons with enlarged pores. Still, these osteons appear only to have a minor contribution to the aging-induced increase in cortical porosity, which mainly appear to be the result of cumulative eroded and formative pores with a non-terminated bone remodeling (Further elaborated in the text).

Importantly, type 2Q osteons are the end result of multiple remodeling transactions i.e. intracortical BMUs, each leaving behind a cement line, which remained visible if not resorbed during subsequent remodeling transactions. In some cases we were able to observe clear remains (cement lines) of up to five remodeling transactions, creating multiple generations of osteons upon each other. Moreover, this supports the concept that a negative BMU balance at each remodeling transaction may cumulative lead to the generation of quiescent osteons with enlarged pores [22,38,41,42], and explain why 7.4% of the type 2Q osteons had a pore diameter > 75 µm, due to an insufficient refilling of their cumulative resorption area, i.e. osteon diameter. Still, not all remodeling transactions may change the pores of type 2Q osteons. Most pores of type 2Q osteons actually have a pore diameter similar to the type 1Q osteons, suggesting that they, more or less, return to their original pore diameter. Some remodeling transactions even seal osteons, forming so-called blind sealed canals or sealed osteons [57–59]. This means that pores of quiescent osteons are not an irreversible bone loss, since it may be sealed by a subsequent remodeling transaction.

4.2. Contribution of type 2Q osteons with enlarged pores to the higher cortical porosity with age

Age-induced cortical porosity is mainly the result of enlarged pores, rather than an increased pore density [23–25]. According to the investigated concept [22,38,43], these enlarged pores accumulating with age and generating the higher cortical porosity should reflect the enlarged pores of type 2Q osteons. This was however not the case. Although the prevalence of type 2Q osteons with enlarged pores (pore diameter > 75 µm) and their contribution to the porosity had a positive correlation with the overall cortical porosity, their contribution to the overall porosity was minor and unchanged with age. This reveals that the enlarged type 2Q osteons reflecting the end result of multiple remodeling transactions with. a negative BMU balance [41,42], as they passes through the plane of the histological section, only have a negligible contribution to the higher cortical porosity with age, at least in ilia of women.

4.3. Magnitude of resorption, formation and the BMU balance between the two remain unchanged during aging

In the present study, we show no overall age-associated changes in the mean osteon diameter, mean wall thickness and mean pore diameter, demonstrating that the BMUs magnitude of resorption, formation and their BMU balance in the ilia of women are in general unchanged with age. This is in direct contradiction with earlier studies of osteons in transiliac biopsies from women, which reported a lower wall thickness, higher pore diameter, and lower or unchanged osteon diameter with age [22,60].

This contradiction may be explained by the fact that these studies [22,60]: i) investigated merely 34 neighboring formative or quiescent osteons of so-called Haversian remodeling systems within the middle two quarters of the cortex of each individual, providing a biased and

Table 1
Reported age-related changed in the quiescent osteons diameter/area, wall thickness and pore diameter/area.

Gender	Skeletal site	n	Correlation with age			Reference
			On.Dm (On.Ar)	W.Th	Po.Dm (H.Ar)	
Women	Iliac	41	–	↓	↑	Brockstedt et al. [22]
		36	↓	↓	↑	Broulik et al. [60]
	Rib	117		–		Pirok et al. [62]
		28	↓		–	Qiu et al. [68]
	Femur dia.	10	↓			Arnold [70]
		33		↑	↓	Singh and Gunberg [63]
		43	↓			Britz et al. [64]
		10	↑	–	↑	Busse et al. [67]
Men	Iliac	23	↑	–	↑	Brockstedt et al. [22]
		32	↑	–	↑	Broulik et al. [60]
	Rib	209		–		Pirok et al. [62]
		37	↓		–	Qiu et al. [68]
	Femur dia.	12	↓			Arnold [70]
		33		↑	↓	Singh and Gunberg [63]
		45	↓			Britz et al. [64]
		13	↑	–	↑	Busse et al. [67]
	Femur Neck	20	–		↓	Tong et al. [66]
	Tibia	33		↑	↓	Singh and Gunberg [63]
	Mandible	52		↑	↓	Singh and Gunberg [63]
Mixed	Iliac	50	–	–	–	Verna et al. [61]
	Rib	130	–			Takahashi and Frost [69]
		27	↓			Goliath et al. [65]
	Femur dia.	19	↓	–	–	Currey [61]
		27	↓			Goliath et al. [65]
	Mandible	50	–	↓	↑	Verna et al. [61]

↑ positive correlation with age; ↓ negative correlation with age; – no correlation with age.

limited selection of osteons; and ii) did not take the presence of type 2 pores/osteons into consideration. Hence, the measured wall thickness and osteon diameter in these studies may have been overestimated, as they may easily have based these estimates on the cement line of older osteons. Moreover, they may also have missed new erosions within pores of existing osteons, overestimating the pore diameter of the quiescent osteons. Here our marking, classification of the pores/osteons and validation by a second observer reduced this risk. This may explain why the same group could not reproduce their findings in ilia, but only in mandible [61], and why similar studies of osteons in ribs, femora and tibiae have observed variable age-related changes in the osteon diameter, pore diameter and wall thickness [62–71] (Table 1). This heterogeneity might be due to difference between skeletal sites and genders (Table 1), but may likely also just reflect different observers and methodologies.

4.4. Non-quiescent pores, not quiescent pores, are the main contributor to a high cortical porosity

Despite the fact the cortical porosity was associated with a higher mean pore diameter in the quiescent osteons due to a decreased wall thickness and unchanged osteon diameter, the contribution of their pores to the porosity actually had a negative correlation with the cortical porosity. On the other hand, the prevalence of non-quiescent pores and their contribution to the porosity had a positive correlation with cortical porosity. This implies that it's not the end result of the BMUs with a terminated remodeling, observable as pores of quiescent osteons, which contribute to a higher cortical porosity, but the non-quiescent pores of ongoing or arrested BMUs that contribute to a higher cortical porosity. A recent complementary study has revealed that these non-quiescent pores primary are enlarged eroded type 2 pores, reflecting intracortical BMUs with a prolonged reversal-resorption phase leading to a delayed bone formation [25]. Here a similar accumulation of eroded surfaces has been reported in cancellous bone of patients with osteoporosis [3,72].

4.5. Limitations of the study

Even though we investigated three times as many quiescent osteons in iliac bone specimens from a similar number of women as in previous studies [22,38], one can argue that the age distribution of the women is a limitation of the study, since it includes only a restricted number of women aged 40 to 60 years. This also made it impossible to investigate the potential additive effect of menopause. Importantly, future studies are needed to validate whether our findings in ilia of women are transferable to other skeletal sites and men.

Of note, one also has to bear in mind that individual intracortical BMUs have been shown to change their osteon diameter, wall thickness and pore diameter dynamically as they progress in an older study 3D-tracing the activities of 20 intracortical BMUs in dogs [58]. In other words, the reported osteon area, the amount of bone formed and the balance between the two reflects only the activities of BMUs at the 2D-level they passed through the plane of the histological section. The same holds true for the resorptive areas position relative to the existing osteons [37], since all intracortical remodeling events must be initiated upon a surface of an existing canal, remodeling the canal of origin (type 2 osteons), while some may branch of and form new canals (type 1 osteons). Future studies systematically 3D-tracing the activities of individual BMUs in human are therefore necessary to understand the dynamics of the BMUs activities and their interception with existing canals.

4.6. Conclusion

Collectively, the present study strongly questions the importance of a negative BMU balance to the higher cortical porosity with age in ilia of women, where this conventional concept was originally established in both cortical and cancellous bone [22,38,44–49]. Still, some intracortical BMUs had a very negative BMU balance when passing though the plane of the histological section, observable as quiescent osteons with enlarged pores, the intracortical BMUs magnitude of resorption, formation, and their BMU balance was not altered with age. Instead, a higher cortical porosity was associated with the accumulation of non-quiescent pores, not pores of quiescent osteons. These non-quiescent pores were recently shown to be primarily cumulative eroded pores upon pores of existing osteons, reflecting BMUs with a prolonged reversal-resorption phase causing a delayed initiation of the subsequent bone formation [5,25,30]. This implies that we need to turn our attention to the reversal-resorption phase and initiation of the bone formation, rather than the magnitude of bone formed when initiated, in order to understand and prevent age-induced cortical porosity. Still, future studies are warranted to validate the controversial findings of this study in ilia of men and other skeletal sites.

Disclosures

All the authors state that they have no conflicts of interest.

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Authors roles: The study was designed by CMA and TLA. The ethical approval and data handling related to the bone specimens was conducted by BE and JL. The analysis was conducted by CMA and TLA, whom also take the responsibility for the integrity of the data analysis. The data was analyzed by TLA and interpreted by CMA, JMD and TLA. The manuscript was drafted by TLA and revised by all authors, whom also approved the final version.

The following are the supplementary data related to this article.

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